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Rapid Communication

The majority of the nucleotides in the top loop of the genomic 3′ terminal stem loop structure are *cis*-acting in a West Nile virus infectious clone

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Abstract

The flavivirus genome RNA terminates with a conserved 3′ stem loop (SL) structure that was shown to be essential for virus replication. A stretch of conserved nts is located in the top loop (TL) of this structure. Mutation of the TL nts (5′ ACAGUGC 3′) in a WNV infectious clone indicated that 3 of the 7 TL nts (5′ ACAGUGC 3′) are critical for virus replication. Mutation of 3 of the other nts reduced the efficiency of virus replication. The four 5′ TL nts are conserved in both mosquito- and tick-borne flavivirus genomes, while the TL 3′ C is conserved in mosquito-borne viruses. The conservation of two or three G–C base pairs in the TL flanking sequences suggests that a stable stem is necessary for precise presentation of the TL sequence. The TL may participate in RNA as well as protein interactions.

Keywords: West Nile virus; 3′ *cis*-acting nucleotides; Infectious clone; 3′ Stem loop structure

Introduction

West Nile virus (WNV) is maintained in nature in a bird–mosquito transmission cycle. Humans and horses are incidental hosts but some infected individuals develop severe disease that can be fatal. Endemic in Africa, the Middle East and Western Asia, particularly India for some time, WNV was first detected in the United States in 1999 and has since become endemic in the Western Hemisphere (Petersen and Roehrig, 2001; Rappole et al., 2000). WNV is a member of the genus flavivirus within the family Flaviviridae. This positive strand RNA virus has a genome of about 11 kb, which serves as the only viral mRNA. The single open reading frame encoded by the viral RNA produces a polyprotein that is cleaved by viral and cellular proteases into three structural and seven nonstructural proteins (Lindenbach and Rice, 2001). The 5′ noncoding region (NCR) is 96 nts in length, while the 3′ NCR is 631 nts (Brinton, 2002). Among the known groups of animal positive-strand RNA viruses, the members of the family Flaviviridae are the only ones with genomes that lack a 3′ poly A tract. The stem loop (SL) structure formed by the 3′ terminal ~80 nts has been predicted based on thermodynamic, co-variation, phylogenic, and chemical data (Brinton et al., 1986; Grange et al., 1985; Proutski et al., 1997; Rauscher et al., 1997). The 3′ structure is conserved among divergent flaviviruses despite the lack of extensive sequence conservation in this region (Brinton et al., 1986; Lindenbach and Rice, 2001). However, there are a few short stretches of conserved sequence within the 3′ SL (Brinton, 1986, 2002; Markoff, 2003; Wengler and Castle, 1986). Two of these conserved sequences are located near the 3′ end of the sequence and another is located in the top loop (TL) region. The four conserved 5′ nucleotides of the top loop (TL) and the C flanking the 5′ side of the TL have previously been designated the conserved pentanucleotide loop (Khromykh et al., 2003; Wengler and Castle, 1986). Several additional conserved sequences have been identified within the flavivirus 3′ NCR upstream of the 3′ SL (Hahn et al., 1987; Markoff, 2003). Deletion of the terminal 3′ SL in a yellow fever virus infectious clone was lethal (Bredenbeek

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et al., 2003) and viruses with 3′ SL chimeras made with WNV sequences inserted into a Den-2 infectious clone grew very inefficiently (Zeng et al., 1998). Although these results demonstrate the functional importance of the 3′ SL, only a few individual *cis*-acting nts within the 3′ SL have so far been identified. It was previously reported that simultaneous substitution of the two 5′ TL nts as well as the 5′ flanking nt completely inhibited replication of a Kunjin replicon (Khromykh et al., 2003). Cell proteins with molecular masses of 52, 84, and 105 kDa were previously shown to bind specifically to the 3′ terminal SL of WNV RNA (Blackwell and Brinton, 1995). The 52-kDa protein was identified as eukaryotic elongation factor 1a (eEF1a) and one major and two minor binding sites on the 3′ SL RNA for this protein were mapped (Blackwell and Brinton, 1997). One of the minor binding sites for eEF1a was located in the TL. Mutation of the major eEF1a binding site in a 3′ SL RNA probe reduced the relative in vitro RNA binding activity of purified mammalian eEF1a by 60%, while mutation of the five 3′ TL nucleotides in the TL (5′ ACAGUGC 3′ to 5′ AC**UCACG** 3′) reduced the binding activity by 20% (Blackwell and Brinton, 1997). We report here the first systematic mutational analysis of each of the WNV TL nts in a WNV infectious clone. The results indicate that the majority of the TL nts are *cis*-acting.

Results

Alignment of flavivirus 3′ nucleotides

The 3′ terminal nts from the genome RNAs of a number of divergent flaviviruses were aligned (Fig. 2B). Consistent with previous reports, three of the 5′ TL nts (5′ **AC**A/C**G**... 3′) and the C located on the 5′ side of this sequence were completely conserved in divergent mosquito-borne as well as tick-borne flaviviruses. These 5 nts have been referred to previously as the pentanucleotide loop sequence (Khromykh et al., 2003; Markoff, 2003; Wengler and Castle, 1986). However, this nomenclature is not accurate for two reasons. First, in all of the flavivirus 3′ SLs, the 5′ flanking C is predicted to base pair with the G flanking the 3′ end of the TL sequence and so is not part of a loop. Second, this name does not take into account the additional highly conserved nts in this region (indicated in bold in Fig. 2B). The C located at the 3′ end of the TL is conserved in the genomes of the mosquito-borne flaviviruses and the 3′ flanking G is completely conserved among all the divergent flaviviruses. Also, at least one and in most cases two additional TL flanking 3′ Cs and flanking 5′ Gs that are predicted to pair are also conserved (Fig. 2B).

Complete genome RNA sequences for WNV (AF260968), Dengue 2 virus (U87411), and yellow fever virus (NC_002031) sequences were folded by Drs. A. Palmenberg and J.-Y. Sgro, University of Wisconsin Madison (data not shown). For each of these genomes, the optimal fold and 100 randomly sampled suboptimal folds showed only a single preferred structure at the top portion of the 3′ terminal SL. The bases in this region were of very low P-num value, meaning the motifs were "well determined" and highly probable (Zuker and Jacobson, 1995). Moreover, the 3′ SLs in the three genome secondary structure folds, were local and without long-range pairing interactions. Also, the structural boundaries were identical to those found when only the 3′ NCRs were folded, indicating the rest of the genome probably has minimum influence on the folding of this region.

Effect of multiple mutations in the WNV TL

Fig. 2A shows the 3′ SL structure formed by the WNV infectious clone sequence as well as the locations of the three binding sites previously mapped for eEF1a. The effect of mutating all or most of the TL sequence in the WNV infectious clone was first investigated. The strategy used to insert mutations into the infectious clone is described in Materials and methods (Fig. 1). The substitution nts were chosen based on the results of M-fold (Version 3.1) analyses (Zuker, 2003). None of the mutations used in this study were predicted to alter the secondary structure of the 3′ SL.

All 7 of the TL nts were mutated (5′ ACAGUGC 3′ to 5′ **GUUCACA** 3′) to generate mutant TL-m1 (Fig. 2C). Full-length mutant RNA was transcribed in vitro using SP6 polymerase and used to transfect monolayers of BHK cells as described in Materials and methods. No viral plaques were detected 72 h post transfection. Culture fluid was harvested and passed sequentially in BHK cells three times. Seventy-two-hour harvests of each of these passages were tested for infectivity by plaque assay. No viral plaques were detected in any of the samples. To determine whether nonplaquing virus was present, RNA was extracted from 250 μl of culture fluid using TRI Reagent (Molecular Research Center, Inc.), serially diluted in twofold increments and the 3′ terminal region was amplified by RT-PCR as described in Materials and methods. No extracellular virion RNA was detected in any of the samples.

Five of the seven nucleotides of the TL were next changed from 5′ ACAGUGC 3′ to 5′ AC**UCACG** 3′ (mutant TL-m2, Fig. 2D). A previous study had shown that mutation of these 5 TL nts reduced the in vitro eEF1a binding activity for a 3′ SL RNA probe by 20% (Blackwell and Brinton, 1997). No plaques were detected 72 h post transfection of BHK cells with this mutant RNA or during three sequential passages and no extracellular virion RNA was detected.

Mutation of the four 5′ conserved TL nucleotides

The four 5′ TL conserved nts were next mutated in pairs or singly. When the first two 5′ nucleotides were mutated (5′ ACAG... 3′ to 5′ **UU**AG... 3′, TL-m3), only pinpoint plaques were observed on the overlaid transfection plate

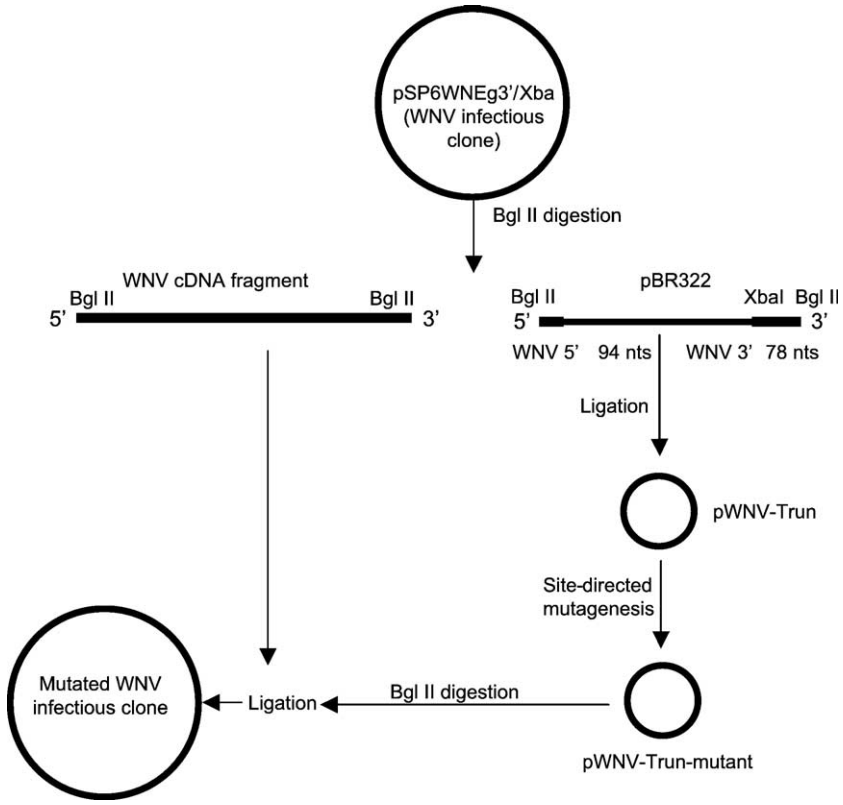


Fig. 1. Infectious clone site-directed mutagenesis strategies. (A) The strategy for generating the shuttle vector, pWNV-Trun, is shown. This shuttle vector was used as a template for Quick-change or PCR mutagenesis as described in Materials and methods. Mutated shuttle vector DNA was ligated to the other *Bgl*II fragment to reassemble the complete clone. (B) A table listing the primers used for RT-PCR amplification of the viral 3′ sequence and for site-directed mutagenesis.

(data not shown). However, when the transfection harvest was titrated in BHK cells, both pinpoint plaques as well as large-sized plaques were observed (Fig. 3C). The diameters of these large plaques were slightly greater than those of the wild type virus (compare Figs. 3B,C). Ten plaques of each size were picked, the RNA extracted and the 3′ terminal region (~300 bp) was amplified by RT-PCR as described in Materials and methods. The PCR products were TA cloned and multiple clones were sequenced in both directions. RNA extracted from the pinpoint plaques contained the introduced mutation, while RNA extracted from the large plaques contained a partial reversion (5′ **UU**AG… 3′ to 5′ **U**CAG… 3′) (Fig. 3G).

A growth curve done in BHK cells with the TL-m3 virus harvested at 72 h from the transfection plate at an MOI of 0.1 indicated that there was a 12 h delay in the detection of the mutant virus as compared to parental virus (Fig. 3F). However, growth was detected by 24 h. Only large plaques were observed on the titration plates for the mutant growth curve samples, suggesting that the revertant (TL-m3R) rapidly out-competed the mutant virus with the pinpoint plaque phenotype. When only the 5′ A was mutated to a U (5′ ACAG… 3′ to 5′ **U**CAG… 3′, TL-m3R*), both small and large plaques were observed on the overlaid transfection plate (Fig. 3D). The large and small plaques were picked and the viral RNA was amplified by RT-PCR and sequenced. The small plaque RNA contained the mutation, while the large plaque RNA had the wild-type sequence. While the TL-m3R* mutant had a small plaque phenotype and rapidly reverted to the wild-type sequence, the original TL-m3R mutant was stable during three passages and had a large plaque phenotype, suggesting the possibility of a second site mutation(s) in the TL-m3R RNA. Other regions of this mutant RNA are currently being sequenced.

The two other conserved 5′ TL nts (5′ ACAG… 3′ to 5′ AC**UU**… 3′) were mutated to generate TL-m4 (Fig. 3A). No viral plaques were produced after transfection of full-length mutant RNA into BHK cells or in culture fluids harvested during three subsequent serial passages in BHK cells. Also, no viral RNA was detected after RT-PCR amplification. Mutation of only the G (5′ ACAG… 3′ to 5′ ACA**U**… 3′, TL-m5) was also lethal (Fig. 3A). No progeny virus was detected either in the transfection wells or in serially passaged samples and no viral RNA was detected in the passaged samples. However, when only the A (5′ ACAG… 3′ to 5′ AC**U**G… 3′, TL-m6) was mutated, both small- and medium-sized plaques were observed on the overlaid transfection plates (Fig. 3E). Plaques of each size were picked and genomic RNA was extracted, amplified by RT-PCR, and sequenced. Viral RNA extracted from the larger size plaques contained a reversion of the **U** to the

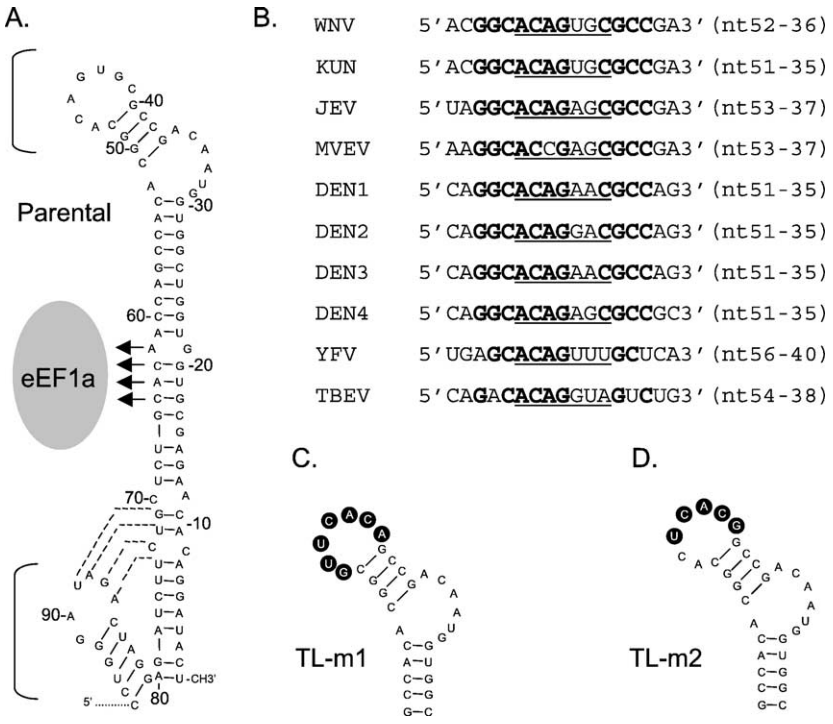
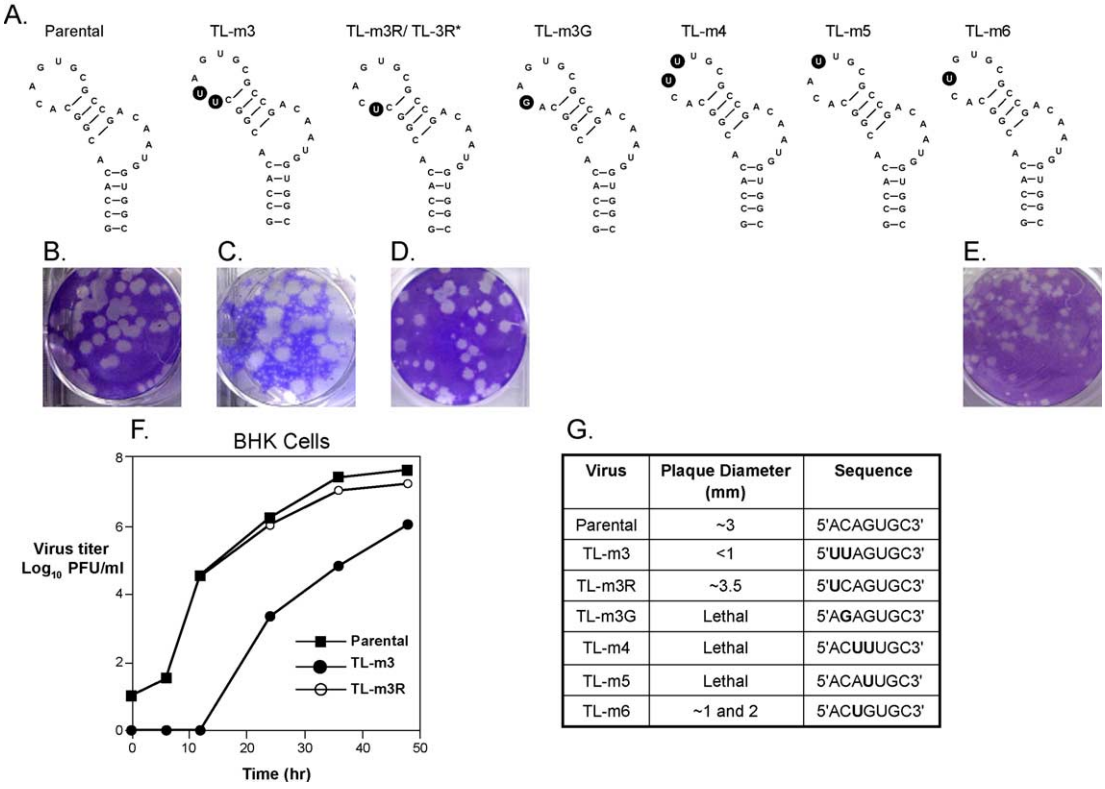


Fig. 2. Multiple site mutations in the TL. (A) WNV infectious clone 3′ SL structure. The previously reported major binding site for eEF1a is indicated by arrows. Minor binding regions are indicated by brackets. (B) Sequence alignment of predicted TL and flanking sequences in different flavivirus RNAs. Accession numbers of the sequences are: Dengue virus serotypes 1–4 (AF311958, U87411, M93130, and AF326573), MVEV (NC_000943), JEV (NC_001437), YFV (NC_002031), TBEV (NC_001672), Kunjin virus (L24512), and WNV E101 (AF260968). Conserved nucleotides are bolded. The TL sequence is underlined. (C) Mutation of all seven of the TL nts (TL-m1) was lethal. (D) Mutation of five of the TL nts (TL-m2) was lethal.



Virus	Plaque Diameter (mm)	Sequence
Parental	~3	5'ACAGUGC3'
TL-m3	<1	5'**UU**AGUGC3'
TL-m3R	~3.5	5'**U**CAGUGC3'
TL-m3G	Lethal	5'**AG**AGUGC3'
TL-m4	Lethal	5'AC**UU**UGC3'
TL-m5	Lethal	5'ACA**U**UGC3'
TL-m6	~1 and 2	5'AC**U**GUGC3'

Fig. 3. Mutation of the 5′ TL nts. (A) The substitutions made to create mutants TL-m3 through TL-m6 are indicated. (B) Plaques produced by parental infectious clone virus. (C) Plaques produced after one passage of TL-m3 in BHK cells. (D) Plaques produced at 72 h after transfection of engineered TL-m3R* RNA. (E) Plaques produced at 72 h after TL-m6 RNA transfection. (F) Virus growth curves in BHK cells. (G) Summary of the data.

parental nt A, while RNA extracted from all the small plaques contained the introduced mutation. The in vitro RNA transcription and transfection steps were repeated twice with the same results indicating the revertant arose within the first 72 h after transfection.

Mutation of the 3′ nucleotides of the TL

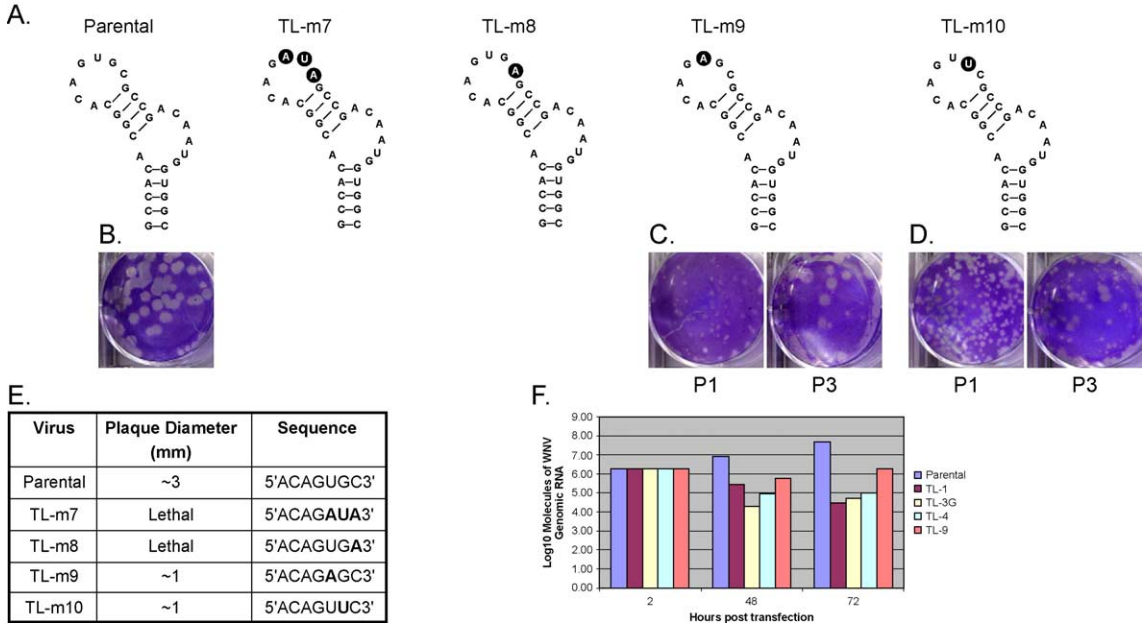
The three 3′ nucleotides of the TL were changed from 5′ . . . UGC 3′ to 5′. . . **AUA** 3′ to generate TL-m7 (Fig. 4A). No plaques were detected on the overlaid well 72 h after transfection or upon titration of the three serially passaged samples and no viral RNA was detected in any of the passaged samples. Mutation of the 3′ C to an **A** (5′. . . UGC 3′ to 5′. . . UG**A** 3′, TL-m8) was also lethal (Fig. 4A).

The TL 3′ U was changed to an **A** (5′ . . .UGC 3′ to 5′. . . **A**GC 3′) to create TL-m9 (Fig. 4A). At 72 h post transfection, only small plaques (~1 mm) were detected. These plaques were opaque, suggesting delayed or reduced cell killing (P1 in Fig. 4C). When RNA was extracted from picked plaques, amplified by RT-PCR and sequenced, only RNA with the introduced mutation was detected. Growth curves showed that the TL-9 mutant grew less efficiently in both BHK and C6/36 cells; the peak virus titer was lower by about $10^{1.5}$ as compared to the parental virus (data not shown). After the third passage, some larger size plaques (~3 mm) were detected (P3 in Fig. 4C). Plaques of both sizes were picked from P3 titration plates, RNA was extracted, amplified by RT-PCR, and sequenced. RNA extracted from the small plaques contained the introduced mutation (5′ **A**GC 3′), while in the RNA extracted from the large plaques, the mutated **A** was replaced with the parental nt U.

The 3′ G was next changed to **U** (5′ UGC 3′ to 5′ U**U**C 3′) creating TL-m10 (Fig. 4A). Only small plaques (~1 mm) were detected 72 h after transfection (P1 in Fig. 4D). When RNA was extracted from picked plaques, amplified by RT-PCR and sequenced, only RNA containing the introduced mutation was detected. Analysis of the growth kinetics of the mutant showed that the TL-m10 virus grew slightly less efficiently in BHK cells and C6/36 cells than the parental virus; the peak virus titer was about $10^{0.5}$ lower than that of parental virus (data not shown). A few larger-sized plaques (~2 mm) were detected after the third passage of TL-m10 virus in BHK cells (P3 in Fig. 4D). Plaques of each size were picked and RNA was extracted, amplified by RT-PCR, and sequenced. The results indicated that the RNA in the small plaques contained the introduced mutation (5′. . . U**U**C 3′), while the RNA extracted from the large plaques contained a reversion of the **U** to the parental nt G.

Analysis of intracellular viral genomic RNA levels

As an initial means of analyzing the functional level affected by the TL mutations, intracellular viral RNA was assayed by real-time RT-PCR at different times after RNA transfection of BHK cells with the various mutated infectious clones as described in Materials and methods. Data were obtained from samples collected from duplicate experiments. The RNA levels in each sample were separately assayed using a primer pair from the NS1 region



Virus	Plaque Diameter (mm)	Sequence
Parental	~3	5'ACAGUGC3'
TL-m7	Lethal	5'ACAG**AUA**3'
TL-m8	Lethal	5'ACAGUG**A**3'
TL-m9	~1	5'ACAG**A**GC3'
TL-m10	~1	5'ACAGU**U**C3'

Fig. 4. Mutation of the 3′ TL nts. (A) The substitutions made to create mutants TL-m7 through TL-m10 are indicated. (B) Plaques produced by parental infectious clone virus. (C) Plaques produced after passages 1 and 3 of TL-m9 in BHK cells. (D) Plaques produced after passages 1 and 3 of TL-m10 in BHK cells. (E) Summary of the data. (F) Assay of intracellular viral genomic RNA levels at 2, 48, or 72 h after transfection of either wild-type or mutated infectious clone RNA by real-time RT-PCR.

and a pair from the 3′ terminal region. Representative data are shown in Fig. 4F. No evidence of viral RNA replication was observed for any of the mutants that did not produce detectable plaques after transfection of BHK cells. Mutants that produced small plaques showed a reduced level of RNA replication as compared to the wild-type infectious clone. These results indicate that the TL mutations did not generate nonplaquing virus and also did not affect viral RNA packaging. The results obtained by Tilgner et al. (in press) showed that mutations in the TL of a WNV replicon do not affect initial translation of the viral RNA.

Discussion

Conservation of sequences among divergent members of a family of RNA viruses indicates their importance in a fundamental process in the virus life cycle, because such sequences must be maintained by viability selection. Three short conserved sequences within the flavivirus 3′ SL were previously identified by sequence alignment (Brinton, 1986, 2002; Markoff, 2003; Wengler and Castle, 1986). One of these sequences, previously named the pentanucleotide loop, is located at the top of the 3′ SL, and includes the nt flanking the 5′ side of the TL, that is predicted to be base-paired, and the four 5′ nts of the TL (Khromykh et al., 2003; Wengler and Castle, 1986). However, additional conserved nts are located on both sides of the TL and in the mosquito-borne viruses, the 3′ C of the TL is also conserved (Fig. 1B).

It was previously reported that mutation of the two 5′ nts of the TL as well as the 5′ flanking C (5′ CACAG. . . 3′ to 5′ **UCU**AG. . . 3′) inhibited Kunjin replicon RNA replication (Khromykh et al., 2003). The contribution of individual mutated nts to this effect was not determined. M-fold analysis predicted that this mutation completely altered the structure of the top of the 3′ SL and positioned a different sequence in the loop at the top of the stem (Davis and Brinton, unpublished data). In the present study, the effect of mutation of individual nts within the TL of the 3′ SL of the WNV genomic was analyzed in a WNV infectious clone. Only mutations that did not change the predicted 3′ SL structure were analyzed. The results showed that three of the seven TL nts (5′ ACAGUGC 3′) are essential for virus replication in vivo. The 5′ C and G are conserved among both mosquito- and tick-borne flaviviruses, while the 3′ C is conserved at least within the JEV and DEN serogroups. Mutation of either the 5′ G or the 3′ C to a **U** was lethal. However, mutation of the 5′ C to a **U** produced viable virus with a pinpoint plaque phenotype.

Although viable virus with a pinpoint plaque phenotype was produced when the conserved 5′ C was substituted with a U in the context of the double mutant TL-m3, substitution of this nt with a G (TL-m3G) was lethal. Substitution of the conserved 5′ G with a U was also lethal. These observations are consistent with the hypothesis that the 5′ TL nts are involved in an RNA–RNA interaction and that a G–U pair can functionally substitute for a G–C pair at least in the double mutant TL-m3 (pinpoint plaque). However, nts in regions involved in RNA–RNA interactions would be expected to co-vary with their binding partners during evolution rather than be strictly conserved. It was previously reported that the TL contains a minor binding site for a cell protein (Blackwell and Brinton, 1997). The strict conservation of the four 5′ nts of the TL may be required to preserve a protein interaction as well as an RNA interaction.

Some of the same mutations reported here were also tested in a WNV replicon (Tilgner et al., in press). Mutations shown to be lethal in the WNV infectious clone (TL-m3G, TL-m4, TL-m5, and TL-m7) completely inhibited replicon RNA replication but not early translation. Two of the infectious clone mutations (TL-6 and TL-9), which produced virus that replicated less efficiently, caused reduced RNA replication when introduced into the replicons. The results obtained with only two mutations differed between the two studies. The engineered TL-m3R* (5′ ACAG. . . 3′ to 5′ **U**CAG. . . 3′) produced viable virus with a pinpoint plaque phenotype that rapidly reverted to wild type. In contrast, when the 5′ A was replaced with a U in the replicon, RNA replication was completely inhibited. Also, in the infectious clone, a U but not a G (TL-m3G) could partially functionally replace the 5′ C in the double mutant TL-m3. In the replicon, replacement of this C with a U completely inhibited RNA replication. It is not known why some mutations that were viable in a WNV infectious clone were not viable in a WNV replicon. The replicon contains a deletion of the majority of the structural gene region. The presence of this region in the infectious clone or the proteins produced from this region may stabilize the TL RNA interactions and rescue these mutations. Another possibility is that the replicon may not remodel the cell environment to facilitate viral RNA replication as quickly or as completely as does the infectious clone.

Mutation of the 3′ C, which is conserved among members of the JEV and DEN serogroups, was lethal. The observation that substitution of this C with a U did not preserve viability suggests that this C may not be involved in an RNA–RNA interaction. Alternatively, it is possible that a G–U pair cannot functionally substitute for a G–C pair at this position. In contrast, mutations that changed one of the two 3′ nonconserved TL nts (5′ ACAG**A**GC 3′, TL-m9 or 5′ ACAGU**U**C 3′, TL-m10) had only a slight negative effect on virus growth. These results suggest that the 3′ C within the WNV TL sequence is essential at least in most of the mosquito borne flaviviruses and that the other two 3′ nts do play a functional role even though they are not conserved.

In each of the flavivirus 3′ RNAs aligned in Fig. 2B, the TL is flanked by a minimum of two G–C base pairs. The JEV and DEN RNAs shown contain three flanking G–C pairs, while WNV and Kunjin have four flanking G–C pairs. This suggests that a stable stem is necessary for the precise presentation of the TL sequence and lends further support to the hypothesis that the TL sequence is an interactive sequence.

Khromykh et al. (2003) suggested that the TL may be a landing pad for the assembly of newly translated replication complex components. This suggestion was based on the findings of Chen et al. (1997), who reported an interaction between the NS3 and NS5 proteins of JEV and the 3′ SL of the viral genomic RNA using low stringency binding reaction conditions. We were not able to demonstrate a specific interaction between either NS3 or NS5 and the 3′ SL RNA in in vitro RNA binding assays (Elghonemy and Brinton, unpublished results). Alternatively, particular cell proteins may interact with the 3′ SL and facilitate the assembly of initiation complexes. Overlapping RNA–RNA and RNA–protein interaction sites in the TL may modulate alternative functional conformations of the 3′ SL.

Materials and methods

Cells

Baby hamster kidney (BHK) 21, strain WI2, cells (Vaheri et al., 1965) were maintained in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) (Atlas) and 10 μg/ml gentamycin (Invitrogen) at 37 °C in a 4.5% CO_2 atmosphere. Mosquito C6/36 cells were maintained in MEM supplemented with 0.1 mM MEM non-essential amino acids, 10 μg/ml of gentamycin, and 10% FCS at 27 °C.

WNV infectious clone

A full-length infectious clone of WNV in pBR322 was kindly provided by Dr. V. Yamshchikov (Yamshchikov et al., 2001). This clone is a chimera. The sequence of the 5′ end through about the middle of NS5 is from the lineage II WNV strain, 956 D 117 3B, while the 3′ part of NS5 and the 3′ NCR are from the lineage I WNV strain, Eg101. This infectious clone was modified by insertion of an A at position 3 from the 3′ end (Perelygin and Brinton, unpublished data). An A at this position was detected previously by direct sequencing of the 3′ end of a highly passaged Eg101 stock (Brinton et al., 1986). Even though the current low passage stock of Eg101 virus in the lab does not contain this A, it was not removed from the chimeric infectious clone because it appeared to have a stabilizing effect in bacteria.

Site-directed mutagenesis

A shuttle vector, pWNV-Trun, which contains the 94 3′ nts and 78 5′ nts from the WNV sequence and the pBR322 plasmid sequence, was generated from the WNV infectious clone DNA by digestion with *Bgl*II, gel purification of the two fragments, followed by ligation of the plasmid containing fragment. Base substitutions were introduced into the 3′ noncoding region (NCR) of the WNV genomic RNA in the shuttle vector by two methods. Mutated primers were used for PCR amplification of the 3′ sequence. These primers also contained terminal *Bgl*II or *Xba*I restriction sites. The PCR fragments were cloned into a TOPO-XL vector (Invitrogen), and selected clones were sequenced using BigDye, version 2.0 or 3.1 (ABI). The *Bgl*II–*Xba*I PCR fragments were then inserted into the shuttle vector replacing the wild-type sequence. Alternatively, mutations were introduced into the shuttle vector by Quick-Change site-directed mutagenesis according to the manufacturer's protocols (Stratagene). The sequences of the primers used for mutagenesis are available from the authors upon request. Selected positive clones were sequenced to confirm the presence of the introduced mutation.

Mutated infectious clones were generated by ligating *Bgl*II-digested pWNV-Trun DNA containing a mutated 3′ SL to the gel purified internal *Bgl*II fragment digested from the wild-type infectious clone (Fig. 2). Clones obtained were first screened by digestion with Aat II to select those with inserts in the correct orientation. The sequences of the 3′ and 5′ regions of the selected clones were confirmed by sequencing.

In vitro transcription of full-length viral genomic RNA

Infectious clone DNA was linearized by digestion with *Xba*I for 2 h at 37 °C, gel purified (Qiagen Gel Extraction), ethanol precipitated, resuspended and (1 μg) was used as the template for in vitro transcription of capped infectious clone RNA done using an AmpliCap SP6 High Yield Message Maker kit (Epicentre) according to the manufacturer's protocol. The transcription reaction was incubated at 37 °C for 2 h.

Transfection of BHK cells with in vitro transcribed WNV genomic RNA

BHK cells were seeded in six-well plates (2×10^5 cells/well) and grown to 90% confluence. The cells were washed once with serum-free medium and then overlaid with transfection mixture containing 1 ml of Opti-MEM (Invitrogen), 6 μl of DMRIE C (Invitrogen), and ~3 μg of the in vitro transcribed full-length WNV genomic RNA, and incubated for 3 h at 37 °C in a 4.5% CO_2 atmosphere. The transfection mixture was replaced with either growth media or 1% SeaKem ME agarose (BioWhittaker Molecular Applications) mixed 1:1 with 2× MEM containing 5% FCS and incubated at 37 °C for 72 h. Media harvested at 72 h was stored at −80 °C. The agarose in overlaid wells was removed and the cells were stained with crystal violet to visualize viral plaques.

RT-PCR amplification of the viral 3′ RNA

The 3′ nts of the viral genome were amplified by RT-PCR. The RT reaction was done at 60 °C using Thermoscript Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Five microliters of the RT reaction were

used in a 50 μl PCR reaction to amplify the cDNA product using the RT-R primer (5′ AGTATCCTGTGTTCTCGCACCACCAGCCAC 3′) and an RT-F primer. For TL-m1 and Tl-m2, the RT-F1 primer (5′ TAGTGGAGACCCCGTGCCAC 3′) was used, while the RT-F2 primer (5′ GCGAAAGGACTAGAGGTTAGAGGAGAC 3′) was used for all of the other mutant RNA amplifications. The conditions used were 95 °C denaturing for 30 s, 48 °C annealing for 15 s, and 72 °C elongation for 30 s for 40 cycles.

Assay of viral growth kinetics

Confluent BHK cultures in 25-cm^2 flasks were infected at an MOI of 0.1 with virus present in culture fluids harvested 72 h after transfection with parental or mutant viral RNA. Adsorption was for 1 h at room temperature. After incubation, cell monolayers were rinsed three times with 5 ml of media to remove unbound virus and 5 ml of MEM containing 5% FCS was added per flask. Samples (0.5 ml) of culture fluid were removed at various times after infection and replaced with 0.5 ml of fresh media. The samples were stored at −80 °C. Virus titers were determined by plaque assay on BHK cells. The virus growth curves as well as the plaque titrations were done in duplicate. Virus titers shown are an average of the duplicate results.

Assay of intracellular viral RNA by real-time RT-PCR

Full-length wild-type or mutant WNV genomic RNAs were generated using the Ampliscribe SP6 Message Maker kit (Epicentre) from infectious clone DNA. Before transfection, the reactions were incubated at 37 °C for 30 min with RNase-free DNase I (1 MBU) to digest the template DNA. BHK cells in six well plates that were approximately 90% confluent were transfected for 2 h with 1 μg of RNA in Opti-MEM containing DMRIE-C (Invitrogen). Total cell RNA was extracted at 2, 48, and 72 h post transfection with TRI Reagent (Molecular Research Center, Inc.). Assays were performed on an Applied Biosystems 7500 real-time PCR system using 400 ng of template RNA with the TaqMan one-step RT-PCR master mix (Applied Biosystems). The primers (5′ GGCGGTTCTAGGAGAAGTCA 3′, 5′ CTCCTGTTGTGGTTGCTTCT 3′) and probe (FRET probe 5′ Fam-TGCACCTGGCCAGAAACCCACACTCTGT 3′ TAMRA) targeted a region within the nonstructural gene NS1 of the WNV genomic RNA. The data were analyzed using the Absolute quantification standard curve software from Applied Biosystems.

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